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Margaret Bruck 10/13/83
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INTRODUCTION

In response to specific stimuli, normal cells undergo a process of cell suicide which has been termed programmed cell death, or apoptosis (1). Triggers for apoptosis include insufficient growth factors, cytokines, steroid hormones and exposure to DNA-damaging agents. The control of cell populations by apoptosis is important for homeostasis and for the removal of damaged, potentially harmful, cells. Since insufficient apoptosis can contribute to cancer cell growth (2,3), elucidation of the molecular mechanism of apoptosis is expected to lead to new anti-cancer treatment strategies. Our long-term goal is to use an understanding of apoptosis to improve the treatment of breast cancer.

Despite intense research in the past few years, the mechanism of cell killing during apoptosis has yet to be fully elucidated. Studies of changes in gene expression during glucocorticoid-induced apoptosis of a murine lymphocyte line led me hypothesize that oxidative stress plays a critical role (reviewed in 4). Oxidative stress reflects an imbalance in the cell between the rates of production and removal of reactive oxygen species (5,6). Mitochondria are a major endogenous source of oxidative stress. Electrons leaking from the mitochondrial respiratory chain are captured by oxygen to produce reactive species. Single electron reduction of oxygen successively generates superoxide anion radicals, hydrogen peroxide and hydroxyl radicals. These molecules cause oxidative stress by damaging DNA, lipids and proteins.

One reported trigger of apoptosis in breast cancer cells is tumor necrosis factor- α (TNF) (7). Results from studies on a limited number of cell types, not including breast cancer cells, implicate oxidative stress in the mechanism of TNF-mediated cell death. Increased levels of reactive oxygen species have been documented in cells following treatment with TNF (8,9) and TNF-mediated death has been inhibited by antioxidants (10). Products of oxidative damage to lipids and DNA have been reported to increase following treatment of murine tumor cell lines with TNF (10,11).

The cells' ability to handle oxidative stress would be expected to influence susceptibility to TNF, given that oxidative stress plays a role in the cell death mechanism. Cellular antioxidant defenses include copper,zinc-superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD), which are located in the cytoplasm and mitochondria, respectively, and convert superoxide anion radical to hydrogen peroxide. Catalase and glutathione peroxidases convert hydrogen peroxide to water. DT-diaphorase (NAD(P)H:quinone (acceptor) oxidoreductase) may also protect against oxidative stress (12). Wong and Goeddel have demonstrated a correlation between manganese superoxide dismutase (MnSOD) gene expression and response to TNF (13); TNF-sensitive transformed cell lines were found to display low or undetectable levels of MnSOD transcripts, while resistant cell lines were found express MnSOD at relatively high constitutive levels. TNF sensitive A375 melanoma (14), human embryonic kidney and ME-180S cervical carcinoma cells (15) were found to be more resistant to TNF when stably transfected with an MnSOD expression vector.

The above studies raise the question of whether TNF-induced apoptosis of breast cancer cells involves oxidative stress. Specific areas to be addressed include examining whether: 1) the status of antioxidant defenses changes with TNF treatment; 2) the cells show signs of oxidative stress; and 3) whether modulating antioxidant defenses alters sensitivity to apoptosis. This project has been aimed at addressing these questions using the human breast adenocarcinoma-derived MCF-7 cell line. Techniques employed in the studies have included northern blot hybridization analyses and biochemical assays for studies of gene expression and enzyme activities, respectively, and stable transfections of antioxidant defense genes in conjunction with measures of apoptosis and cell viability following TNF treatment of the cells.

Hypothesis, specific aims and significance of this project

The **hypothesis** being tested here is *that oxidative stress plays a critical role in the mechanism of apoptosis induced by treatment of human breast cancer cells with TNF*. The **specific aims** for the project are to: 1) use Northern blot hybridization analyses and enzyme assays to define the extent of changes in the cellular antioxidant defense, after treatment of breast cancer cells with TNF; 2) determine whether breast cancer cells that have been transfected with expression vectors encoding antioxidant defense genes are resistant to TNF-induced apoptosis; 3) determine whether the induction of anti-sense antioxidant defense genes is sufficient to cause apoptosis of breast cancer cells, and 4) investigate the effects of oxidative stress, in breast cancer cells, on redox-sensitive transcription factors.

The proposed project is **significant** to the treatment of breast cancer because: 1) it is likely that defects in the apoptotic pathway contribute to the conversion of normal breast tissue to malignant cancers; 2) induction or restoration of apoptosis has promise as a novel approach to treating breast cancer; 3) an understanding of the underlying molecular mechanism is needed to guide the rational development of apoptosis-based breast cancer treatments, and 4) the proposed experiments test a specific hypothesis relevant to the mechanism of apoptosis in breast cancer cells.

BODY

EXPERIMENTAL METHODS

Cell culture. MCF-7 cells were maintained at 37°C under 5% CO₂ in either RPMI (Irvine Scientific, Santa Ana, CA) or low glucose Dulbecco's Modified Eagle's Medium. Cells used for northern blot hybridization analyses and biochemical analyses were grown in RPMI. Transfected cells were maintained in DMEM. Except for RPMI, all cell culture reagents were obtained from Gibco BRL (Gaithersburg, MD). Media was supplemented with L-glutamine, 5% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin. Cultures were tested periodically and confirmed to be mycoplasma-free. Recombinant human TNF was purchased from Boehringer Mannheim (Indianapolis, IN).

MnSOD expression and activity in detached vs. adherent cells. MCF-7 cells were plated in twenty 150 mm tissue culture dishes. After an overnight incubation, TNF was added to sixteen of the dishes (16 ng/ml final concentration). Four dishes were left untreated, as controls. After 8, 24, 32, 48, 56 and 72 h, the culture media was removed from each dish and centrifuged at 900 x g to collect any detached cells. The media was returned to the appropriate dish. Pellets collected from the control and TNF-treated dishes were pooled separately, washed once with PBS and stored at -80°C. After the media was removed at 72 h, the adherent cells were collected by trypsinization, washed once with PBS and frozen. RNA was extracted from half of the samples and used to analyze MnSOD mRNA levels by northern blot hybridization analyses, as described previously (16,17). In order to compare MnSOD activity between detached and adherent cells, the rest of the samples were thawed, resuspended in 100 mM triethanolamine-diethanolamine, pH 7.4 and lysed, while on ice, using 3-10 sec. bursts from a VC40 Ultrasonic Liquid Processor (Sonics & Materials, Inc., Danbury, CT). The lysates were centrifuged at 100,000 x g and then dialyzed to remove small interfering substances. Samples were stored at -80°C until assayed. Total SOD activity was measured as described by Paoletti et al. (18). The method is based on the inhibition of a superoxide-driven NADH oxidation. The final assay mixture contained 88 mM

triethanolamine-diethanolamine, pH 7.4, 2.4 mM EDTA, 1.2 mM MnCl_2 , 280 μM NADH and cell lysate; the reaction was initiated by the addition of 2-mercaptoethanol to a final concentration of 939 μM . MnSOD activity was determined after incubating the cell lysates at 4°C for 1 h with 50 mM sodium cyanide (19). The final concentration of sodium cyanide in the assay mixture was 5 mM.

Detection of reactive oxygen species with dichlorodihydrofluorescein diacetate. MCF-7 cells were seeded into 96-well plates; a separate plate was used for each timepoint in the experiment. After two days of growth, each plate was divided into thirds. One third of the wells were left untreated and one third received TNF (final concentration of 20 ng/ml). The remaining third were used as a positive control; H_2O_2 was added to these wells just before measuring fluorescence (see below). Following 1, 3, 5.5 and 24 h of TNF treatment, the cells were washed twice with PBS to reduce interference from phenol red in the culture media. Half of the TNF-treated samples in the plate were exposed to dichlorodihydrofluorescein diacetate for 20 min at 37°C; each of these wells received 100 μl of phenol red-free RPMI media containing 5% fetal bovine sera, 50 μM dichlorodihydrofluorescein diacetate (Molecular Probes, Inc., Eugene, OR) and 5% DMSO. To control for background fluorescence, the remaining half of the TNF-treated samples received 100 μl per well of phenol red-free RPMI media containing 5% fetal bovine sera and 5% DMSO. The untreated samples in the dish were similarly divided and exposed to either dichlorodihydrofluorescein diacetate- or DMSO-containing media. At the end of the 20 min. incubation, the media in all the wells was replaced with phenol red-free RPMI media containing 5% fetal bovine sera. At this time, H_2O_2 was added to half of the untreated wells to a final concentration of 600 μM . By cleaving the dichlorodihydrofluorescein diacetate taken up by cells, H_2O_2 serves as a positive control for detection of the fluorescent compound. Fluorescence was measured at excitation and emission wavelengths of 485 nm and 530 nm, respectively, using a 7620 Microplate Fluorometer with a 7010 Stabilized Light Source (Packard Instrument Co. Inc., Meriden, CT). Fluorescence readings measured with DMSO alone were subtracted from the readings taken of corresponding samples containing dichlorodihydrofluorescein diacetate.

Sensitivity of DT-diaphorase transfectants to TNF. Stable DT-diaphorase transfectants of the MCF-7 cell line were established in the second year of the project. Control transfectants, carrying the neomycin resistance gene only, were also established. To compare the TNF sensitivity between transfectants, the cells were plated in 96-well plates at three cell densities: 3,000; 6,000 and 12,000 cells per well. Each dilution of cells was plated in seven wells. Twelve wells in each microtiter plate were not seeded with cells, to correct for background absorbance in the sulfarhodamine B assay. After a two hour incubation at 37°C, TNF was added to half of the wells at each cell density for a final concentration of 20 ng/ml. Incubation was continued for an additional 48 h. Cell viability was then determined using the protein-binding dye, sulfarhodamine B, as described previously (20). Briefly, the cells in the microtiter plates were washed two times with PBS. Ice cold TCA (100 μl) was added to each well and the plates were incubated at 4°C for 30 min. The TCA was removed by washing the plates \approx 4 times with tap water and excess water was removed by air-drying. Thirty microliters of sulfarhodamine-B was added to each well and the plates were incubated at room temperature for 20 min. After 3 rinses with 1% acetic acid, the plates were left to dry before solubilizing the protein-bound sulfarhodamine B dye in Tris base (50 mM, 100 μl /well) by shaking the plate on a rotary platform for 5 min. Absorbance was read at 540

nm using a microplate reader and corrected for background absorbance by subtracting the average value from the wells with media alone. To test for statistically significant differences in TNF responses between transfectants, the log values of the corrected absorbance readings were plotted against the number of cells plated. The purpose of the log transformation was to linearize the data. The difference in the regression lines between the -TNF and +TNF treatments was then determined for each transfectant. Finally, an Analysis of Covariance model was used to test for significant variation in the difference between the -TNF and +TNF regression lines for different transfectants.

Analysis of gene expression in MCF-7 transfectants. Confluent cultures of neo^r and DT-diaphorase transfectants were harvested and total RNA was extracted using CsCl gradients, as described previously (16). Northern blot hybridization analyses (17) were used to assess DT-diaphorase message levels in the samples. To verify that mRNA detected on the northern blot was derived from the transfected DT-diaphorase gene, RT-PCR were performed. RNA samples (2.5 µg) extracted from MCF-7 and DT-15 cells were incubated with 0.5 ng Oligo (dT)₁₂₋₁₈, 0.5 mM each dNTP, 5 mM DTT and 200 units Superscript II Reverse Transcriptase in buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂ (final concentrations) in a total reaction volume of 20 µl. This first strand cDNA synthesis was carried out for 50 min. at 42°C. The Reverse Transcriptase was then inactivated by heating at 70°C for 15 min. PCR reactions were carried out using 1 µl of the first strand reaction, 2 ng of each DT-diaphorase primer (see below), 250 µM each dNTP, and 1 U Taq polymerase in buffer containing 60 mM Tris-HCl, pH 8.5, 1.5 mM MgCl₂ and 15 mM (NH₄)₂SO₄ in a final reaction volume of 50 µl. PCR mixes were set-up with everything except cDNA template and were heated for 5 min. at 80°C before starting the reaction by the addition of template. The parameters for the PCR were 2 min. at 94°C, followed by 30 cycles of 1 min. at 94°C, 2 min. at 55°C and 3 min. at 72°C with an extension of 3 secs. per cycle and ending with a 7 min. incubation at 72°C. Oligo (dT), dNTP mix, Reverse Transcriptase, Taq polymerase and appropriate buffers were obtained from Gibco BRL (Gaithersburg, MD). The primers used in the PCR were designed to amplify rat- and human-specific DT-diaphorase fragments of approximately 450 and 600 bp, respectively. The primer sequences are:

Common upstream primer: 5'-GGCTGGTTTGAGAGAGTG-3';

Rat-specific downstream primer: 5'-GTCGGCTGGAATGGACTTG-3';

Human-specific downstream primer: 5'-GCACGAATACGGTCGATTC-3'.

Amplification was carried out using a 480 DNA Thermocycler from Perkin Elmer (Norwalk, CT). Samples of the PCR reactions (10 µl) were subjected to electrophoresis through 2% agarose and visualized by staining with ethidium bromide.

Sensitivity of catalase transfectants to oxidative stress. Stable catalase transfectants had been generated by introducing a mammalian expression vector encoding neomycin resistance and rat catalase cDNA under the control of the CMV into MCF-7 cells. To test sensitivity to oxidative stress, cells were plated in 96-well plates at a density of 6,000 cells/well. After an overnight incubation, H₂O₂ was diluted and added to the cells for final concentrations of 400, 600, 800, 1200 and 1800 nM. Some samples were left untreated. After 15 min., the media was removed and replaced with fresh media. Incubation was continued at 37°C for 72 h and viability was then assessed using the SRB assay. To test sensitivity to xanthine/xanthine oxidase, cells were similarly plated and treated with 3, 15, or 60 µg/ml xanthine oxidase and 100 µg/ml xanthine. After 72 h, SRB assays were performed.

RESULTS AND DISCUSSION

Expression of MnSOD in detached vs. adherent cells. Studies completed in the first three years of the project demonstrated increased expression and activity of manganese superoxide dismutase (MnSOD) with TNF treatment of MCF-7 cells (21). The expression of other antioxidant defenses, CuZnSOD, catalase and thioredoxin, was not altered by TNF treatment. There appeared to be no change in catalase activity in the treated cells and glutathione peroxidase activity was undetectable.

A useful characteristic of apoptosis in adherent cell population is that the apoptotic cells separate from their neighbors and the substratum and are released into the media. In our studies on the antioxidant defense status with TNF treatment, at the end of the treatment period we had collected the cells that were floating in the media and combined them with the adherent cells. Thus, we did not know whether the changes we had observed in MnSOD expression and activity were occurring to the same extent in the apoptotic vs. the surviving cells. During this past year, we examined MnSOD expression and activity in the two separate cell populations. MCF-7 cells were treated with 1,600 U/ml TNF for three days. Floating cells were collected 8 hr after TNF treatment and then twice a day for the next two days. Floaters were collected, for the final time, on the third day and the adherent cells were then harvested. The separate populations were then analyzed for MnSOD expression and enzyme activity. The results of these experiments (Fig. 1), demonstrated that the change in MnSOD was most pronounced in the adherent cell population.

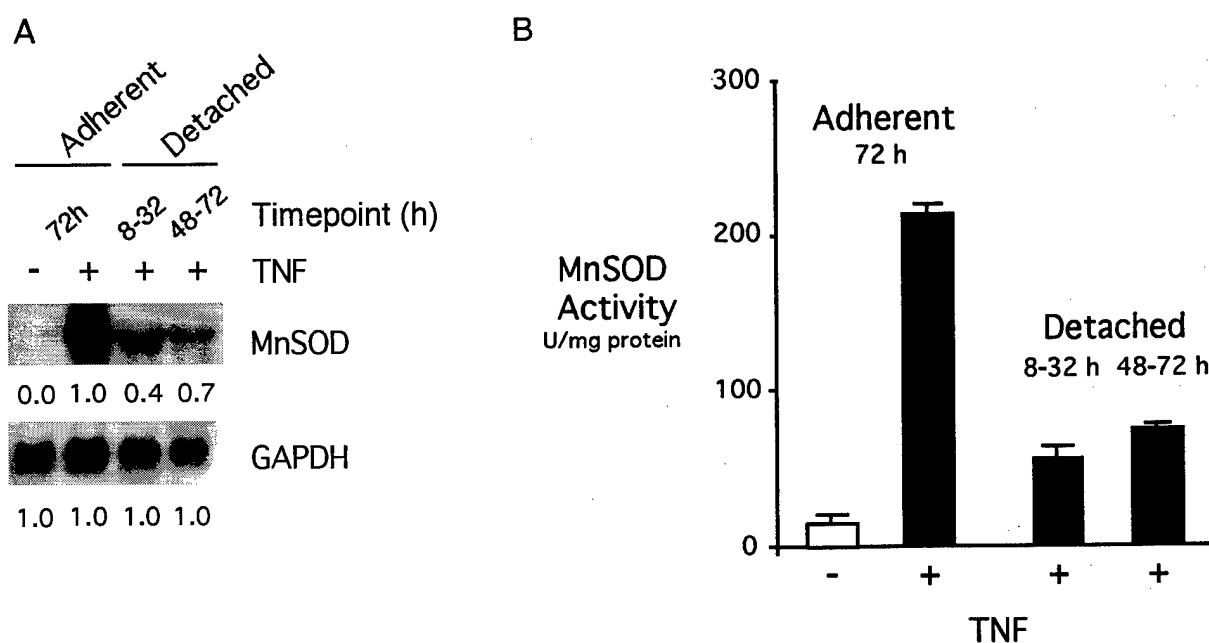


Figure 1. MnSOD expression and activity in detached and adherent MCF-7 cells after TNF treatment. Cells were incubated in the absence or presence of 16 ng/ml TNF. Detached cells were collected from the media after 8, 24, 32, 48, 56 and 72 h. At 72 h, cells still adhering to the plates were collected. Samples were processed for: A) northern blot hybridization analysis of MnSOD expression; and B) MnSOD activity measurements (see Experimental Methods). The values indicated under the northern blots represent intensities relative to the adherent, TNF-treated sample, corrected for the relative intensity of the signal seen with the GAPDH probe used as a control for sample loading and transfer.

Taken together, our studies show a selective increase in MnSOD with TNF-treatment of MCF-7 cells. Since the increase in MnSOD occurs predominantly in cells surviving TNF treatment, this suggests that superoxide anion radicals generated in mitochondria contribute to the mechanism of TNF-induced apoptosis. We propose that the cells undergoing apoptosis are those in which MnSOD activity does not increase rapidly enough.

Evidence of oxidative stress in TNF-treated MCF-7 cells. Another approach to determining whether oxidative stress is important for TNF-induced apoptosis of MCF-7 cells is to test for increased levels of reactive oxygen species or oxidative damage. Last year, we measured malondialdehyde levels following TNF treatment as an indication of lipid peroxidation. We did not detect significantly increased levels of malondialdehyde following treatment. This negative result could have been due to the relatively low sensitivity of the malondialdehyde assay, or the small numbers of cells undergoing apoptosis at any one time. It should be possible to get around either of these problems by using a more sensitive assay for reactive oxygen species or oxidative damage.

This past year, we tried detecting increased levels of reactive oxygen species using dichlorodihydrofluorescein diacetate (H₂DCFDA). This is a colorless, non-fluorescent cell-permeable dye that been used as a sensitive assay for oxidative stress in a number of systems (22,23). Once inside the cell, H₂DCFDA is deacetylated by esterases. The resulting compound, dichlorodihydrofluorescein, is converted to a fluorescent dye upon attack by oxidizing species in the cell. To look for reactive oxygen species in MCF-7 cells, we incubated samples in the absence or presence of 20 ng/ml TNF for 1, 3, 4.5 and 24 h and then exposed the cells to H₂DCFDA for 20 min. at 37°C. The increased fluorescence in the TNF-treated samples suggest that the levels of reactive oxygen species in the cells has increased by 4.5 h of treatment (Fig. 2). At 24 h, 3-fold higher fluorescence was detected in the treated vs. untreated cells.

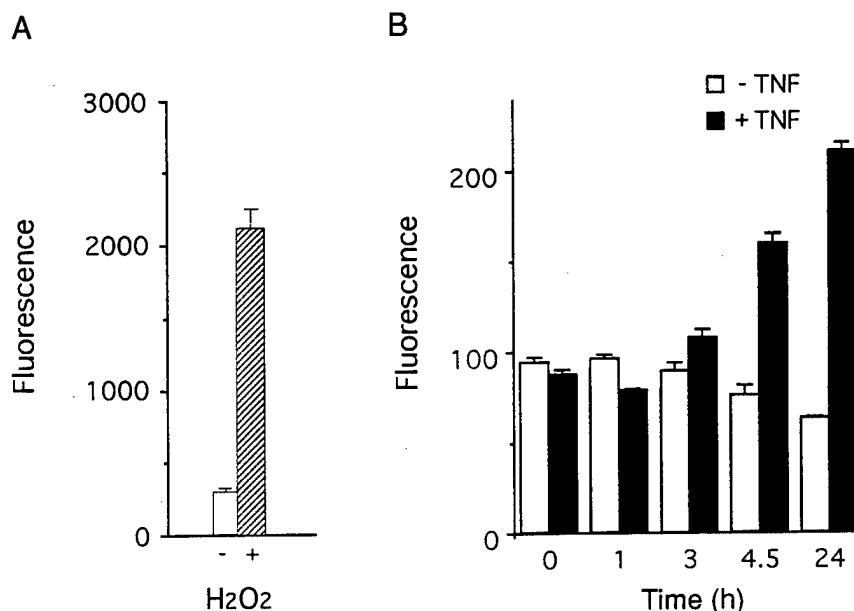


Figure 2. Increased reactive oxygen species in TNF-treated cells. A) MCF-7 cells were exposed to H₂DCFDA, followed by 600 μ M H₂O₂. B) MCF-7 cells were incubated in the absence or presence of TNF (20 ng/ml) for the indicated amount of time and then exposed to H₂DCFDA. Levels of reactive oxygen species were assessed by measuring fluorescence.

Role for mitochondria in TNF-induced apoptosis. Our findings of a selective increase in MnSOD and elevated levels of reactive oxygen species during TNF-induced apoptosis are consistent with a role for mitochondria in the mechanism of cell killing. MnSOD is localized to the mitochondria and catalyzes the conversion of superoxide anion radicals to H_2O_2 . Superoxide anion radical could be generated in mitochondria by leakage from the electron transport chain. Using specific inhibitors, Fiers and co-workers (24,25) found that they could either inhibit or enhance TNF-mediated cytotoxicity in murine fibrosarcoma cell lines. Inhibitors of early points in the electron transport chain, NADH or succinate dehydrogenase, protected against TNF. In contrast, cell killing was increased by blocking electron transport further down. These findings are consistent with TNF treatment leading to increased formation of reactive oxygen species following leakage of electrons from the electron transport chain. The protective effect of MnSOD and our observed lack of an increase in peroxidases suggest that superoxide radical is critical to cell killing. Indeed, TNF-resistant L929 cells have a decreased capacity to produce superoxide anion radical in response to TNF (9). Especially significant to our studies is the recent report by Li and Oberley (26) demonstrating that MCF-7 cells transfected with MnSOD expression vectors have increased resistance to TNF.

The most damaging reactive oxygen species in the cell is thought to be the hydroxyl radical. It can be produced from superoxide anion radical and H_2O_2 in the Haber Weiss reaction or from superoxide anion radical and transition metals in the Fenton reaction. An increased rate of removal of superoxide anion radical would decrease either of these reactions and thereby reduce the possibility of generating hydroxyl radicals. Increased MnSOD activity could raise H_2O_2 levels in mitochondria, particularly since we have found that peroxidase activity did not increase with TNF treatment of MCF-7 cells. Work by Goosens et al. suggests that increased H_2O_2 may be scavenged by glutathione (27). These researchers have shown that when cells are first depleted of glutathione by treatment with diethyl maleate, TNF treatment leads to a much greater increase in the level of reactive oxygen species.

Results from several different laboratories provide evidence for the importance of mitochondria to TNF-mediated cell killing and apoptosis. Mitochondria appear to be necessary for a cytotoxic response to TNF. This is suggested from studies of a panel of myelogenous leukemia ML-1a clones isolated following growth in the presence of ethidium bromide to inhibit mitochondrial DNA synthesis (28). Within the panel of clones, resistance to TNF-induced DNA fragmentation is correlated with reduced respiration. Sensitivity to TNF is restored when mitochondria are introduced back into the resistant clones by fusion with platelets. Studies of the mechanism of apoptosis in response to other agents are consistent with a role for mitochondria. During the development of a cell-free model of apoptosis, a mitochondrially-enriched cell fraction was discovered to be an essential component (29). Cytochrome c has been identified as a factor which can cause apoptotic changes in extracts of untreated cells (30). This protein, a component of the electron transport chain, is released into the cytoplasm of HeLa cells treated with staurosporine to induce apoptosis (30). Why cytochrome c is released from the surface of the inner mitochondrial membrane into the cytoplasm and the importance of this event to the mechanism of apoptosis remain to be seen. Overexpression of Bcl-2 blocks cytochrome c release in CEM cells treated with UVB, etoposide, staurosporine or H_2O_2 (31) and in HL-60 cells treated with staurosporine or etoposide (32). Since Bcl-2 overexpression prevents apoptosis and, in particular, activation of cysteine proteases downstream of cytochrome c release (31,32), this suggests that the release of cytochrome c is indeed a significant event in the apoptotic program.

Elucidating the relationship between increased production of reactive oxygen species in mitochondria, cytochrome c release and activation of cysteine proteases in TNF-treated cells is critical to understanding the mechanism of apoptosis. Studies aimed at identifying proteins downstream of the TNF receptor have led to models in which engagement of the receptor results in the recruitment of FLICE/MACH1 (33-35), a protein with homology to cysteine proteases (36,37). Given that this model reflects the sequence occurring *in vivo*, it remains to be shown how the recruited, latent protease is activated in cells that are triggered to die by apoptosis in response to TNF. Considering the evidence supporting a role for mitochondria and reactive oxygen species in the mechanism of TNF-mediated cell killing, it may be that increased superoxide production leads to release of cytochrome c from mitochondria and subsequent activation of the CPP32-like protease recruited by engagement of the TNF receptor. This possibility could be an important area of future study with the MCF-7 cells.

Development of a statistical approach to assessing TNF responses. The goal of the second specific aim of the project is to test the importance of oxidative stress to the mechanism of TNF-induced apoptosis through analysis of cells expressing increased levels of antioxidant defense enzymes. Completion of this Specific Aim is dependent upon a reliable method for comparing apoptosis of different transfectants. Last year, after comparing several different approaches to measuring apoptosis, we concluded that scoring apoptosis by microscopical examination of stained samples was more reliable than TUNEL staining or an ELISA for DNA fragmentation. Subsequently, we realized that counting apoptotic cells microscopically is too labor-intensive to be practical for comparing a number of different transfectants. Additional time was spent this past year to develop a practical statistical approach for comparing the response of different cells to TNF.

One of the methods that we had tried for assessing responses to TNF was the sulfarhodamine B (SRB) assay. This is an indirect method for apoptosis. It measures protein content, which is known to correlate well with viability. The advantage of the SRB assay is that it can be done using microtiter plates. A number of cell types can be compared at the same time and many replicates for each cell type can be included within each experiment. This made the SRB assay attractive to us for testing for statistical significance. The experiments completed in the first year of the project had determined that the loss of viability with TNF treatment of MCF-7 cells is due to increased apoptosis. Thus, it seemed reasonable to develop a way to use the SRB assay to compare TNF responses between MCF-7-derived cell types.

As we worked more with the SRB assay, it quickly became clear that plating density was an important factor. We had plated cells at several different densities in order to insure that we would be in the linear range of absorbances when reading the results. When we calculated the ratio of the absorbance reading of the +TNF samples over the -TNF samples, we noticed that this value was not constant across the cell densities (data not shown). This was seen even if all of the absorbance readings were within the linear range. One solution to this problem would be to calculate the ratios using the same cell density for each cell type. This turned out to not be feasible, however, as we found it technically impossible to plate the same number of cells for different cell types or on different days (based on absorbance readings of the -TNF samples).

For help in solving the above problem and developing a statistical approach to using the SRB assay, we consulted with the Biometry Department at the Arizona Cancer Center. After reviewing our data, the statisticians were able to recommend a statistical approach that could be used for different cell densities. When analyzing our data they

had confirmed that cell density was a significant factor in the TNF response. The approach that they recommended took this into account by applying an Analysis of Covariance model (with plating density as a covariant) in the statistical analyses. Details on the approach are given in the Experimental Methods section. Briefly, the different transfectants are seeded into microtiter dishes at several different densities and incubated in the absence or presence of TNF for 2-3 days. The SRB assay is then performed. The log of the absorbance readings obtained in the assay are plotted against the cell density plated. For each transfectant, the TNF response is calculated as the difference between the slope of the lines from the +TNF and -TNF values. The Analysis of Covariance model is then applied to test for significant differences in the TNF response between transfectants.

Variation in treatment start time does not influence the TNF response. In the course of working out a method for comparing TNF responses between transfectants, we also examined whether the response was influenced by the amount of time between plating the cells and application of TNF. Previous experiments had included an overnight incubation of the cells prior to addition of TNF. To test whether applying TNF at other timepoints made a difference in the response, MCF-7 were plated and incubated for 1, 3, 6 or 24 h. TNF was then added to a final concentration of 50 ng/ml. After an additional 24 and 48 h, the cells were collected by trypsinization and the viable cell number was determined based on exclusion of Eosin Y dye. The results of this experiment (Table 1) shows no influence of earlier additions of TNF.

Table 1. Effect of Treatment Start Time on Response to TNF

Treatment Start Time After Plating (hr)	Percent Viability (24 h)	Percent Viability (48 h)
1	75	45
3	81	40
6	75	50
24	80	43

DT-diaphorase transfectants show increased sensitivity to TNF. Using the approach recommended by the Biometry Department, we compared TNF responses between the DT-diaphorase transfectants that were established in the first years of the project. For these experiments, we used a 2 day treatment with 20 ng/ml TNF. The response of two clones transfected with the neomycin resistance vector only was compared and found not to be significantly different (data not shown). Therefore, the results for these two were combined and compared to the TNF response for the DT-diaphorase transfectants, DT-1, DT-6, DT-9 and DT-12. In each case, the DT-diaphorase transfectants were found to be significantly more sensitive to TNF (Fig. 3 and Table 2).

The increased sensitivity of the DT-diaphorase transfectants to TNF would seem in contradiction to the protection afforded to MCF-7 cells by overexpression of MnSOD. The apparent contradiction is most likely explained by the unique ability of DT-diaphorase to catalyze reactions that can either protect or damage the cell (38). DT-diaphorase (also referred to as NAD(P)H:quinone oxidoreductase) catalyzes the two-electron reduction of a diverse group of substrates that includes quinones (39).

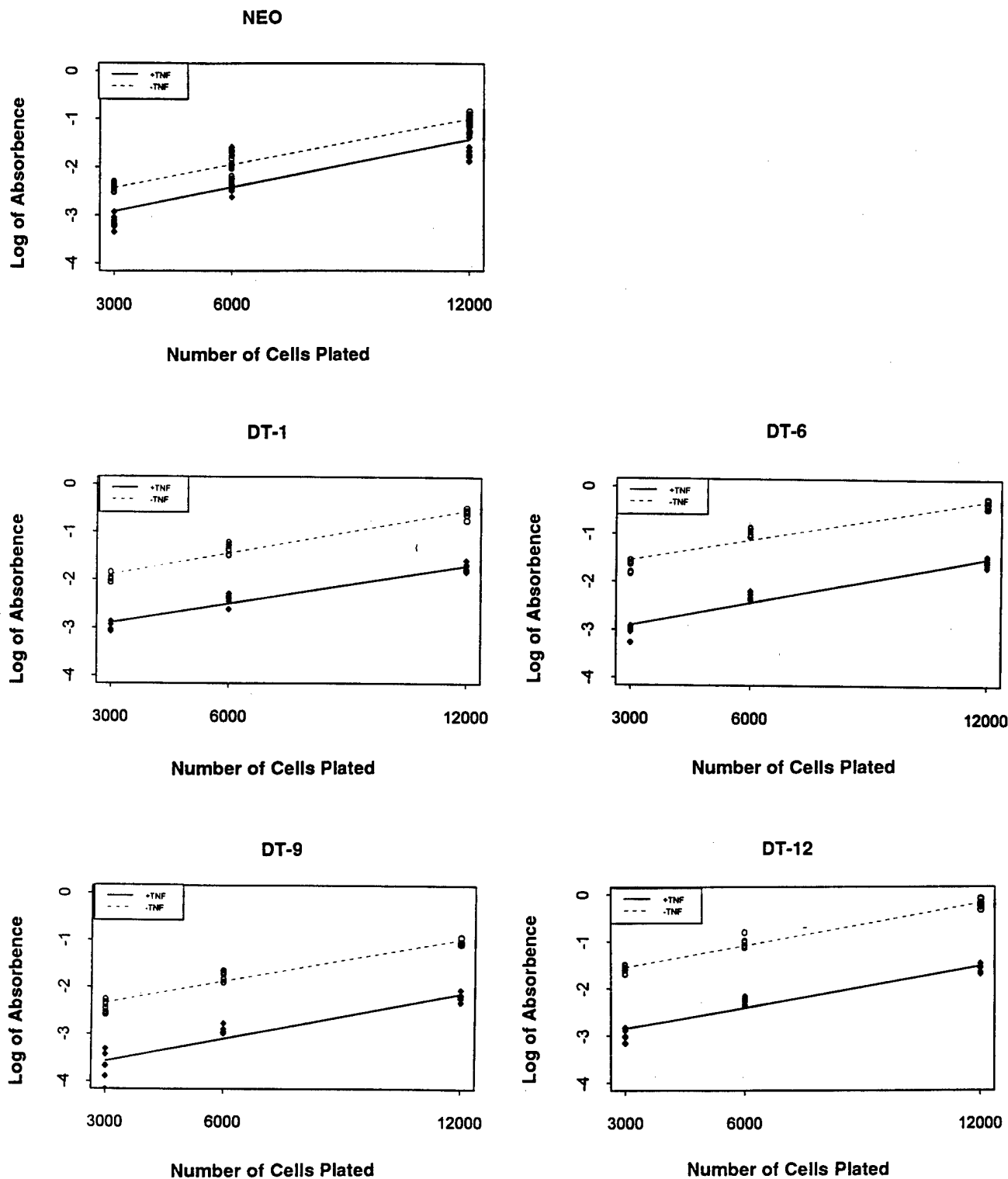


Figure 3. TNF response of neo^r and DT-diaphorase transfectants. Transfectants were seeded at 3000, 6000 or 12,000 cells/well in 96-well plates. Half of the cells were treated with 20 ng/ml TNF for 48 h. Viability was then determined using the sulfarhodamine B assay. Graphs show the log of the absorbance readings from the samples (7 per treatment for each cell type) plotted against the number of cells plated. The difference between the -TNF and +TNF regression lines is an indication of TNF sensitivity.

Table 2. Difference in TNF response between transfectants

Transfectant	Decrease in Log of Protein Content Between -TNF and +TNF Samples	Significance Between neo ^r Clone and Transfectant*
neo ^r	0.46 ± 0.06	-
DT-1	1.06 ± 0.04	0.0095
DT-6	1.28 ± 0.05	0.0001
DT-9	1.21 ± 0.06	0.0001
DT-12	1.32 ± 0.04	0.0001

* P-value from the test for significant difference in decrease of protein content

With certain quinone substrates, DT-diaphorase activity protects the cell by limiting their concentration in the cells. This is beneficial because these quinones are also metabolized by one-electron reductases through a mechanism that generates reactive oxygen species. Acting on other types of substrates, DT-diaphorase harms the cell by generating unstable hydroquinones (40). These compounds either autooxidize, generating reactive oxygen species, or rearrange to nucleophilic forms that can alkylate DNA. Thus, the chemical nature of the substrate and product involved in reactions catalyzed by DT-diaphorase determines whether or not the enzyme activity is beneficial to the cell. Given that the increased sensitivity of DT-diaphorase transfectants to TNF is indeed significant to the mechanism of cell killing, this suggests that the enzyme may act on cellular substrates to generate reactive species that promote apoptosis.

Characterization of DT-diaphorase expression in the transfectants. Having observed an effect of DT-diaphorase over-expression on TNF sensitivity of MCF-7 cells, we have continued with the characterization of the transfectants. We were first interested in determining whether DT-diaphorase message levels corresponded to previous measurements of enzyme activity. During the previous project year, DT-diaphorase levels in MCF-7, neo6, DT-9 and DT-12 and DT-15 cell lysates were measured at 178 ± 1 , 71 ± 6 , 4901 ± 77 , 1071 ± 59 and 7400 ± 560 nmol/mg/min, respectively. The relative levels of DT-diaphorase message in these cells, as seen by northern blot hybridization analysis, fit well with the enzyme activities (Fig. 4A). The most intense signal is seen for RNA from DT-15 cells, followed by DT-9 and DT-12 cells. No signal is seen for the MCF-7 and neo6 samples. We also worked out a RT-PCR-based method to test whether the increased message levels seen in the northern blot analysis were due to expression from the transfected rat gene. A common upstream PCR primer, which would allow amplification from both rat and human sequence, was identified along with downstream primers that are species-specific (see Experimental Methods). All three primers were used together in RT-PCR reactions with RNA purified from MCF-7 cells and the DT-15 transfectant. As shown in Fig. 4B, only one band was amplified from the MCF-7 sample, while two bands were seen in the DT-15 sample. The sizes of the amplified fragments (≈ 600 bp for human and ≈ 450 bp for rat) are consistent with what was expected. The increased intensity of the rat-derived fragment, relative to the human-derived sequence, is consistent with more message being expressed from the transfected vs. the endogenous gene.

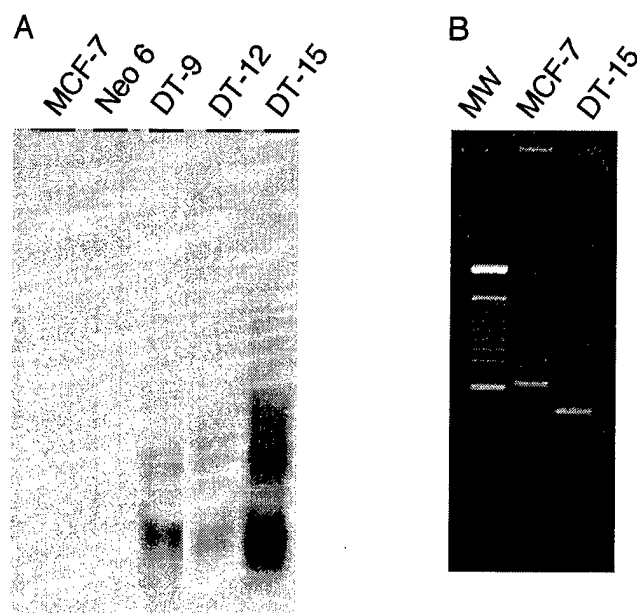


Figure 4. Characterization of DT-diaphorase expression in transfectants. Total RNA was extracted from the indicated cell types and used for (A) northern blot hybridization or (B) RT-PCR analyses of DT-diaphorase expression. The RT-PCR amplifies an ≈ 600 bp fragment from human sequences and an ≈ 450 bp fragment from rat sequences. MW: 100 bp molecular weight ladder; the darker band in the middle is 600 bp.

Analysis of catalase transfectants. Stable MCF-7 catalase transfectants were isolated in the previous project year; this year, we made some progress towards their characterization. The vector which was used for these transfection encodes for neomycin resistance and rat catalase cDNA under the control of the CMV promoter. Catalase enzyme activities in the MCF-7 parent, *neo^r* and catalase transfectants are given in Table 3. In contrast to the DT-diaphorase transfectants, the fold increase in enzyme activity seen in the catalase transfectants is relatively small. It is possible that greatly increased catalase levels in cells are not compatible with growth.

Our next step in the characterization of the catalase transfectants is to determine whether they have altered resistance to oxidative stress. An initial experiment was done, in which the cells were exposed to a range of concentrations of H_2O_2 and xanthine oxidase in the presence of excess xanthine. The latter treatment generates an extracellular burst of superoxide anion and H_2O_2 . Sulfarhodamine B assays were used to assess viability 3 days after the treatment. We have performed this experiment only once so far. The results do not suggest a clear difference between the catalase and control transfectants (data not shown). Before testing the sensitivity of the transfectants to TNF, we would like to be assured that the cellular redox state in these cells has indeed been altered. The experiment with H_2O_2 and xanthine/xanthine oxidase needs to be repeated. We will also modify the design of the experiment in order to be able to better test for significant differences between control and catalase transfectants.

Table 3. Analysis of MCF-7 catalase transfectants.

Cell Line	Catalase Activity ($\mu\text{mol}/\text{mg}/\text{min}$)
MCF-7 parent	6.69 ± 0.25
NeoA	9.61 ± 0.87
NeoB	9.49 ± 0.36
Catalase1	21.90 ± 0.13
Catalase2	12.04 ± 0.19
Catalase3	11.54 ± 0.57
Catalase4	11.21 ± 0.34
Catalase6	7.58 ± 0.33
Catalase7	13.26 ± 0.54
Catalase8	6.32 ± 0.09
Catalase9	11.38 ± 0.45

Antisense transfections. The third specific aim of the project is to use antisense methods to test the role of antioxidant defenses in TNF-induced apoptosis. This aim was to have been completed in the third and fourth year of the project. The experimental approach for this specific aim was to be decided by what was learned about the TNF-sensitivity of the MCF-7 cells stably transfected with antioxidant defenses genes. Given that we demonstrated increased resistance to apoptosis in transfectants with enhanced expression of an antioxidant defense gene, the plan was to determine whether reduced expression of the gene increased sensitivity to TNF. We had hoped to make progress on this specific aim during the last year. Once it was clear that we would need the whole year to complete an assessment of TNF responses in the DT-diaphorase transfectants, we requested a no cost extension of the grant period, in order to work on this unaddressed Specific Aim. The request has been granted. We plan on testing the effect of expression of antisense DT-diaphorase sequences on the sensitivity of MCF-7 cells to TNF. In light of our results to date, we would anticipate finding increased resistance.

Activation of redox-sensitive transcription factors. The goal of the fourth specific aim of the project is to examine the effect of TNF treatment on redox-sensitive transcriptions factors, specifically NF- κ B and AP-1. A series of papers in *Science* reported on the role of NF- κ B in TNF-induced apoptosis (41-43). Previously it had been hypothesized that NF- κ B activation leads to the expression of genes which contribute to the mechanism of apoptosis (44). Indeed, this had been our reasoning for looking at NF- κ B activation in TNF-treated MCF-7 cells. Surprisingly, the *Science* papers demonstrated that loss of NF- κ B function increases *sensitivity* to apoptosis. This suggests that the activated transcription factor regulates the expression of genes which *protect* the cells from apoptosis.

MCF-7 cells were not used in the three studies mentioned above, raising the question of whether NF- κ B affords protection against TNF in these cells. This question was addressed in a study by Cai et al. (45), reported this last year. Using electrophoretic mobility shift assays after a 90 min. treatment with 50 ng/ml TNF, the investigators found

that NF- κ B was indeed activated in MCF-7 cells. They subsequently isolated clones stably transfected with a mutated I κ B α gene. The mutated I κ B α protein acts in a dominant-negative fashion to inhibit NF- κ B activation. In the stable transfectants, TNF treatment did not result in NF- κ B activation or increased expression of MnSOD. In contrast to the reports mentioned above, there was no difference in the cytotoxic effect of TNF between the control and mutant I κ B α transfectants.

During the past year, we still made no progress on the transcription factor study as we concentrated on completing the analysis of the transfectants. Given that a study has now provided evidence questioning the importance of NF- κ B activation in TNF-induced apoptosis of MCF-7 cells, it is unclear what will be gained by carrying out studies of NF- κ B activation as planned for the fourth Specific Aim. The effect of TNF on AP-1 activation in MCF-7 cells has not been addressed, however. This is work that we plan on tackling during the next year.

CONCLUSIONS

Based on the results of the project obtained to date, we conclude that in the MCF-7 human breast adenocarcinoma cell line:

- 1) There is a selective increase in the antioxidant defense enzyme MnSOD;
- 2) The increase in MnSOD is most pronounced in those cells which remain viable after exposure to TNF; this is consistent with other reports of a protective effect of MnSOD against TNF-induced cytotoxicity;
- 3) The increase in MnSOD, an enzyme localized to mitochondria that acts on superoxide anion radicals, is consistent with studies implicating the importance of mitochondria to the mechanism of apoptosis;
- 4) Sulfarhodamine B assays performed with samples plated in 96-well plates at various densities and analyzed by an Analysis of Covariance statistics model is a practical approach to comparing TNF responses between different MCF-7-derived cell types;
- 5) Varying the amount of time, up to 24 h, between plating MCF-7 cells and beginning TNF treatment does not affect the response seen;
- 6) MCF-7 cells stably transfected with a rat DT-diaphorase cDNA and expressing increased DT-diaphorase message and higher enzyme levels have increased sensitivity to TNF;
- 7) Since increased sensitivity to TNF was seen for DT-diaphorase transfectants expressing a range of higher enzyme levels, this suggests that a threshold level may be sufficient for altering TNF sensitivity;
- 8) Further studies are needed to confirm that the increased TNF sensitivity seen for the DT-diaphorase transfectants is indeed a reflection of the mechanism of cell killing.

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